The Role of *Notch 1* Activation in Cardiosphere Derived Cell Differentiation

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Cardiosphere derived cells (CDC) are present in the human heart and include heterogeneous cell populations of cardiac progenitor cells, multipotent progenitors that play critical roles in the physiological and pathological turnover of heart tissue. Little is known about the molecular pathways that control the differentiation of CDC. In this study, we examined the role of *Notch 1*/J kappa-recombining binding protein (*RBPJ*) signaling, a critical cell-fate decision pathway, in CDC differentiation. We isolated CDC from mouse cardiospheres and analyzed the differentiation of transduced cells expressing the Notch1 intracellular domain (*N1-ICD*), the active form of Notch1, using a terminal differentiation marker polymerase chain reaction (PCR) array. We found that Notch1 primarily supported the differentiation of CDC into smooth muscle cells (SMC), as demonstrated by the up-regulation of key SMC proteins, including smooth muscle myosin heavy chain (*Myh11*) and *SM22 α* (*Tagln*), in *N1-ICD* expressing CDC. Conversely, genetic ablation of *RBPJ* in CDC diminished the expression of SMC differentiation markers, confirming that SMC differentiation CDC is dependent on *RBPJ*. Finally, in vivo experiments demonstrate enhanced numbers of smooth muscle actin-expressing implanted cells after an injection of *N1-ICD*-expressing CDC into ischemic myocardium (44 ± 8 /high power field (hpf) vs. 11 ± 4 /high power field (hpf), $n=7$ sections, $P<0.05$). Taken together, these results provide strong evidence that *Notch1* promotes SMC differentiation of CDC through an *RBPJ*-dependent signaling pathway in vitro, which may have important implications for progenitor cell-mediated angiogenesis.

Introduction

RESIDENT CARDIAC PROGENITOR CELLS (CPC) are multipotent cells that are found in the adult heart [1–4], possess the capacity to differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells (SMC), and play an important role in cardiac cellular homeostasis and myocardial regeneration in acutely injured [1,2,4,5] and scarred myocardium [6,7]. Cardiosphere derived cells (CDC), a major source of cardiac multipotent stem cells, express GATA4 and Nestin and can differentiate into cardiomyocytes and typical neural crest-derived cells, including neurons, glia, and SMC [8]. CDC have proven functional benefits in cardiac regeneration and can be isolated from human atrium or endomyocardial biopsy specimens [9–16]. However, the mechanisms that regulate the differentiation of CDC into cardiovascular cells are poorly understood.

The Notch1 signaling pathway is evolutionarily conserved from invertebrates to vertebrates and is involved in cell-fate decisions during development [17–20]. Notch1 signaling is activated via juxtacrine binding of an adjacent cell's Delta-

like or Jagged ligands with the Notch receptor. On activation, the Notch1 intracellular domain (N1-ICD) is released from the membrane receptor via enzymatic cleavage. The N1-ICD can function as a transcriptional activator, translocating into the nucleus and forming a DNA-binding complex with J kappa-recombining binding protein (RBPJ) to control the downstream effectors of Notch1 signaling, such as *Hes1*. Notch1 signaling is important in regulating CPC differentiation depending on the cell type. For example, activated Notch1 can promote the early specification of CPC to a highly proliferative myocyte phenotype via *Nkx2.5* upregulation [21]. However, Notch signaling also regulates the SMC differentiation of epicardium-derived cells [20], which play a crucial role in the formation of the coronary vasculature.

CDC are comprised of progenitors from different sources and, therefore, constitute a heterogeneous cell population. To date, little is known about the role of Notch1 signaling in CDC differentiation. In this study, we tested the hypothesis that Notch1 activation can promote cardiac lineage cell differentiation of CDC using novel terminal differentiation polymerase chain reaction (PCR) array technology. We

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found that Notch1 strongly promotes SMC differentiation of CDC in vitro, and that an injection of *N1-ICD*-expressing CDC leads to enhanced numbers of implanted cells differentiating into SMC in a mouse myocardial infarction model.

Materials and Methods

CDC culture

Mouse CDC were isolated as previously described [22] using a protocol approved by the Institutional Animal Care and Use Committee of the University of Cincinnati. Briefly, adult mouse hearts were minced to 1 mm³ in size and plated on laminin-coated dishes for 2 weeks. The round, phase-bright migrating cells were harvested and filtered with 40 µm cell strainers to avoid heart tissue contamination. Cells were cultured in poly-D-lysine-coated dishes for an additional 2 weeks until cardiospheres formed, which were collected and cultured in fibronectin-coated dishes. Lin[−] cells were isolated from the cardiospheres through the use of a hematopoietic Lin-depletion cocktail (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's protocol. The selected CDC cells were cultured and maintained in complete media containing Dulbecco's modified Eagle's medium (DMEM)/F12, 10% fetal calf serum, 200 mM L-glutamine, 55 nM β-mercaptoethanol, and 1% MEM non-essential amino acids (Invitrogen Corporation, Carlsbad, CA). CDC were passaged 10 times at 5-day intervals before use in subsequent experiments. We also isolated CDC from a mouse strain carrying a *loxP*-flanked *RBPJ* gene (*RBPJ* *fl/fl*) with the permission of Dr. Tasuku Honjo (Kyoto University, Kyoto, Japan), as previously described [23,24]. *RBPJ* *fl/fl* CDC cells were infected with adenovirus expressing Cre-recombinase for the deletion of *RBPJ*.

Flow cytometry

Flow cytometric analyses were performed as previously described [22]. Briefly, cultured cells were blocked with 5% bovine serum albumin, and stained with a panel of antibodies including Sca-1, c-kit, and CD90 (BD Biosciences, San Jose, CA), or isotype controls (Vector Laboratories, Burlingame, CA), respectively. The cells were subjected to flow cytometry using a BD LSRII flow cytometer and BD FACS-DivaTM software.

Plasmid construct and generation of recombinant adenoviruses

The adeno-backbone plasmid (*pAdtrack-CMV-N1-ICD*) was produced by amplifying 2.4-kb of human *N1-ICD* cDNA from *EF.h1CN1.Ubc.GFP* (Addgene 17626) via PCR with primers containing *NotI* and *XhoI* restriction sites on the ends (Forward PCR primer: AAGGAAAAA GCGGC CGC GCCACC ATGCGGCGGCAGCATGGCCA. Reverse

PCR primer: CTAG CTCGAG TTAATTGAACGCCTCCGG GA.). The *N1-ICD* fragment was subcloned into the *NotI*-*XhoI* site of the *pAdtrack-CMV* vector (Addgene plasmid 16405). Recombinant adenovirus *Adtrack-CMV-N1-ICD* and *Adtrack-CMV* were generated by using the AdEasy-1 expression system, as previously described [25]. Briefly, parental empty *pAdtrack-CMV* bearing *GFP*, and *pAdtrack-CMV-N1-ICD* vectors bearing *N1-ICD* and *GFP*, were first linearized with *PmeI*, and then, introduced into BJ5183-AD-1 electroporation competent cells (Agilent Technologies, La Jolla, CA) by electroporation. Recombinant adenoviral plasmids were recovered from *E. coli* and introduced into AD-293 cells by Eugene HD (Roche Applied Science, Indianapolis, IN). The recombinant virus was propagated in AD-293 cells. The viruses were purified and concentrated using the Adeno MINI Purification ViraKit (VIRAPUR, LLC, San Diego, CA). Cells seeded on dishes were infected with adenovirus at a multiplicity of infection of 500.

Quantitative reverse transcription-polymerase chain reaction and PCR arrays

Total RNA was extracted from adenovirus infected CDC^{GFP} and CDC^{N1-ICD} using the RNeasy Plus Micro kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. Approximately 1 µg of total RNA was used for cDNA synthesis using the BluePrint 1st Strand cDNA Synthesis Kit (Takara Bio, Inc., Madison, WI) following the manufacturer's instructions. The cDNA synthesized was used to perform quantitative PCR using Mouse Terminal Differentiation Markers RT² Profiler PCR Array (catalog no. PAMM-048A) on an Mx3000P Real-Time PCR System (Agilent Technologies, Santa Clara, CA) using the SensiMix SYBR kit (Bioline, Tauton, MA) according to the manufacturer's instructions. The data were analyzed using the RT² profiler PCR Array Data Analyzer template provided by Sa-bioscience. Gene expression was compared between CDC^{GFP} and CDC^{N1-ICD}, and genes that showed more than 2.5-fold difference in expression were identified. We verified results using primers listed in Table 1. For each sample, the gene of interest was normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) before the calculation of relative fold up- or down-regulation in the transcription levels compared with CDC^{GFP}.

Western blotting

Cell lysate supernatants were prepared, resolved on a 7%–12% sodium dodecyl sulfate bis-tris gel, and transferred to an Immobilon® FL PVDF membrane (Millipore, Billerica, MA). For Odyssey technology, the membrane was blocked with Odyssey blocking buffer (LICOR Biosciences, Lincoln, NE) and probed with rabbit anti-Transgelin (1:500, 22kda, SC-50446; Santa Cruz Biotechnology, Santa Cruz,

TABLE 1. REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION PRIMER LIST

Gene	Forward	Reverse
<i>Tagln</i>	5-TCTAATGGCTTTGGGCAGTTTGGC-3	5-TTTGAAGGCCAATGACGTGCTTCC-3
<i>Myh11</i>	5-TGAGCTCAGTGACAAGGTCCACAA-3	5-GGAAGCCACATCTTTGGCCAGTTT-3
<i>Hes1</i>	5-CAACACGACACCGGACAAACCAAA-3	5-TGGAATGCCGGGAGCTATCTTTCT-3

CA), rabbit anti-MYH11 (H-44) (1:500, 200kda, SC-98705; Santa Cruz Biotechnology), mouse anti-GAPDH (1:2,000, MAB374; Millipore), and then, IRDye 680 goat anti-rabbit IgG or IRDye 800 Goat anti-mouse IgG at 1:5,000 (LICOR Biosciences). Probed blots were scanned using an Odyssey infrared imager.

Immunofluorescence staining

CDC cells infected with Adeno-empty vector or Adeno-N1-ICD were plated on 8-well chamber slides (BD Biosciences) and fixed with 4% paraformaldehyde. After blocking non-specific binding with 10% goat serum in phosphate-buffered saline, the cells were incubated with rabbit anti-Transgelin (1:50; Santa Cruz Biotechnology), rabbit anti-MYH11 (H-44) (1:50; Santa Cruz Biotechnology), rabbit anti-smooth muscle actin (SMA, 1:100; NeoMarkers, Fremont, CA), and rabbit anti-activated Notch1 antibodies (1:100; Abcam) at 4°C overnight, washed 3× in phosphate buffered saline with Tween 20, and incubated with Alexa 555-conjugated anti-rabbit secondary antibodies (Invitrogen). Slides were mounted using VECTASHIELD HardSet Mounting Medium with DAPI (Vector Laboratories). The staining was analyzed by a Zeiss 710 Laser Scanning Microscope (Carl Zeiss, Thornwood, NY).

Cell labeling, myocardial infarction, and intramyocardial cell delivery

CDC were genetically engineered to express Adeno-N1-ICD and Adeno-GFP. Male C57/BL6 mice (2-month-old) were anesthetized with ketamine/xylazine (100 mg/kg/10 mg/kg, i.p.) and mechanically ventilated. Myocardial infarction was induced via ligation of the left anterior descending coronary artery 2 mm from the tip of the normally positioned left atrium as previously described ($n=6$) [22]. Ten minutes after ligation, a 20 μ L solution containing 5×10^5 cells in DMEM was injected intramyocardially 1 mm above

the ligation site. Animals were handled according to approved protocols and animal welfare regulations of the Institutional Animal Care and Use Committee of the University of Cincinnati.

In vivo differentiation of Adeno-N1-ICD modified CDC

Two weeks after surgically induced myocardial infarction and intramyocardial injection of CDC, mouse hearts were harvested, embedded in optimal cutting temperature compound, snap frozen, cut into 5- μ m sections, and immunostained with anti-cardiac troponin I (1:100, SC-15368; Santa Cruz Biotechnology, Inc.), anti-von Willebrand factor (1:100, F3520; Sigma), or anti-SMA (1:100, RB-9010-P; NeoMarkers) antibodies. Primary antibodies were detected via secondary staining with goat anti-rabbit Alexa Fluor 555-conjugated. Nuclei were counterstained with both DAPI (Vector Laboratories) and Draq5. The staining was analyzed by a Zeiss 710 Laser Scanning Microscope (Carl Zeiss).

Statistical analyses

Values are presented as mean $\pm$ standard deviation, as indicated in the legend. Comparison was evaluated by Student's *t*-test between 2 groups and 1-way analysis of variance among multiple groups. In all analyses, $P < 0.05$ was considered statistically significant.

Results

In vitro studies

Isolation and phenotypic characterization of CDC. Cardiospheres were grown from enzymatically digested adult hearts, and the CDC were isolated using a hematopoietic lineage-depletion cocktail (Fig. 1A). Surface marker expression was profiled by flow cytometry. About 80% of the

FIG. 1. Isolation, expansion, and phenotypic characterization of cardiosphere derived cells (CDCs). **(A)** Isolation and expansion of CDC. (1) cardiac explants were cultured for 2 weeks, (2) round, phase-bright cells migrating from the primary culture of mouse ventricular explants, (3) cardiosphere formation, (4) expansion of CDC. **(B)** Flow cytometric analyses of CDC for expression of the cell surface markers c-kit, Sca-1, and CD90. Color images available online at www.liebertonline.com/scd

sorted cells were positive for Sca-1 expression, 62% cells were positive for CD90, and 4% were positive for c-kit (Fig. 1B). These data indicate that CDC represent a subpopulation of cells that are positive for Sca-1 and CD90.

CDC differentiation induced by overexpression of constitutively active N1-ICD. To assess the differentiation of Notch1-activated CDC, we analyzed the expression of a number of terminal differentiation marker genes that cover 13 organs, using the PCR array. From the genes analyzed, the expression of many cardiovascular markers, including SMC (*Tagln* and *Myh11*), endothelium markers (*Pecam1* and *Nrp1*), cardiomyocyte markers (*Nppa* and *Myh7*), and chemokine receptors for stem cell homing (*CXCR4*), were significantly upregulated in N1-ICD overexpressing CDC (>2.5-fold, Fig. 2). Among all the upregulated genes, *Tagln* and *Myh11*, the SMC markers, were the most strongly upregulated, indicating that SMC differentiation is the main outcome of *Notch1*-activated CDC. Interestingly, Notch1 activation resulted in the downregulation of genes in 5 other systems, including markers for osteoblasts, cholangiocytes, beta cells, oligodendrocytes, and podocytes. We performed PCR using primer sets distinct from the commercial array, which confirm the significantly increased *Tagln* and *Myh11* mRNA expression in *Notch1*-activated CDCs (Fig. 3A).

Both *Tagln* and *Myh11* protein expression is induced by overexpression of constitutively active N1-ICD. Due to posttranscriptional regulation, mRNA expression does not always lead to corresponding increases in protein accumulation [26]. Thus, we evaluated whether *Tagln* and *Myh11* mRNA up-regulation is accompanied by an expression of the cognate proteins. Accordingly, cells infected with the Adeno-N1-ICD vector or Adeno-empty vector were subjected to Western blot with anti-*Tagln*, anti-*Myh11*, and anti-N1-ICD antibodies. Western blot showed that *Tagln* and *Myh11* levels were upregulated by N1-ICD overexpression (Fig. 3B),

FIG. 2. Heat map of cardiovascular genes identified as significantly altered in *Notch1*-activated CDC, using reverse transcription (RT)-polymerase chain reaction (PCR)-based mouse Terminal Differentiation Marker system. Color images available online at www.liebertonline.com/scd

FIG. 3. The constitutively active form of Notch1 intracellular domain (N1-ICD) indiscriminately up-regulates both *Tagln* and *Myh11* mRNA and protein expression in CDC. (A) CDC were infected with adeno-empty vector or N1-ICD-expressing vector. Forty-eight hours after the infection, total RNA was extracted, and RT-PCR analysis was performed using the primers indicated in Materials and Methods. Note the marked induction of *Tagln*, *Myh11*, and human N1-ICD mRNA. (B) Up-regulation of both *Tagln* and *Myh11* protein in CDC overexpressing N1-ICD. Western blot assay was performed using specific anti-*Tagln* antibody, anti-*Myh11* antibody, and anti-GAPDH antibody. The experiments were repeated thrice. RT-PCR, reverse transcription-polymerase chain reaction.

demonstrating that N1-ICD can induce SMC differentiation of CDC. In addition, as depicted in Fig. 4A–F, immunofluorescent staining revealed that *Tagln* and *Myh11* expression was clearly detected only in the cytoplasm of *Notch1*-activated CDC.

***Tagln* and *Myh11* expression in CDC is RBPJ dependent.** To test the hypothesis that *RBPJ* is required for SMC differentiation induced by overexpression of N1-ICD, we employed the MEF cell line OT11, which carries homozygous mutations at the *RBPJ* loci, and its parental wild-type cell line, OT13. The *RBPJ* $-/-$ (OT11) and WT (OT13) fibroblast cell lines are reliable model systems that study *RBPJ* dependent signaling in cell differentiation. Overexpression of N1-ICD by adenoviral-mediated gene transfer induced both *Tagln* and *Myh11* (27-fold) gene expression in OT13 cells (Fig. 5A, B). In contrast, overexpression of N1-ICD failed to induce either *Tagln* or *Myh11* gene expression in OT11 cells (Fig. 5A, B). The known target of Notch, was used as positive control.

To test the hypothesis that endogenous Notch signaling is required for SMC differentiation of CDC (in the absence of overexpression of N1-ICD), we employed *RBPJ* $-/-$ CDC, which carry a homozygous deletion of the *RBPJ* loci. *RBPJ* (fl/fl) CDC were used as a control. *RBPJ* $-/-$ CDC displayed significantly reduced *Tagln* and *Myh11* expression compared with *RBPJ* fl/fl control (Fig. 5D, E), confirming that differentiation of CDC into SMC depends on endogenous Notch signaling.

In vivo studies

Differentiation of Sca-1+ cells in injured myocardium. Our previous studies demonstrated the multipotent capacity of Sca-1+ CDC, which can differentiate into cardiomyocytes, endothelial cells, and SMC in vivo [22]. To investigate the effects of overexpression of N1-ICD in CDC in vivo, we

FIG. 4. Tagln and Myh11 protein expression in CDCs overexpressing *N1-ICD*. Cells were infected with adeno-*N1-ICD* or adeno-empty vector, and immunofluorescence assay was performed using specific anti-Tagln, anti-Myh11, or anti-*N1-ICD* antibody. (A–C) CDC infected with adeno-empty. (D–F) CDC infected with adeno-*N1-ICD*. Color images available online at www.liebertonline.com/scd

injected *N1-ICD*-CDC, or *GFP*-CDC as a control, intramyocardial in syngenic mice after inducing myocardial infarction. Donor cells were detected in the myocardium by laser confocal microscopy 2 weeks after cell injection. As compared with *GFP*-CDC control, *N1-ICD*-CDC yielded more cells that stained positively for SMA ($44 \pm 8/\text{high}$

power field (hpf) in *N1-ICD*-CDC vs. $11 \pm 4/\text{hpf}$ in *GFP*-CDC, $n=7$ sections, $P<0.05$, Fig. 6A, B). Notably, *N1-ICD* modified CDC maintained their multipotency in vivo, also differentiating into cardiomyocytes (Fig. 6C, Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/scd) and endothelial cells (Fig. 6E).

FIG. 5. J kappa-recombining binding protein (*RBPJ*) mediates the increased Tagln and Myh11 expression induced by Notch1 activation. (A, B) OT13 and OT11 cells were infected with adeno-empty or adeno-*N1-ICD* at a multiplicity of infection (MOI) of 500. Forty-eight hours later, total RNA was extracted, and RT-PCR analysis was performed. Note the high-level induction of *Tagln* and *Myh11* mRNA by adeno-*N1-ICD* only in OT13 cells, not in OT11 cells. These experiments were repeated thrice. (C) *Hes1*, the known target of *Notch1*, was used as a positive control. (D, E) *RBPJ* (*fl/fl*) CDC cells were infected with adeno-*Cre* at an MOI of 500. Forty-eight hours later, total RNA was extracted, and RT-PCR analysis was performed. Note the significantly reduced level of *Tagln* and *Myh11* mRNA in *RBPJ* ($-/-$) CDC compared with *RBPJ* (*fl/fl*) CDC. These experiments were repeated 4 times. Color images available online at www.liebertonline.com/scd

FIG. 6. In vivo differentiation of Adeno-*N1-ICD* modified CDC. One month after surgical MI and cell injection, hearts were harvested and sectioned, then sections were immunostained for the lineage-specific proteins (red) cardiac troponin I (cTnI), von Willebrand factor (vWF), or smooth muscle actin (SMA); nuclei were counterstained with Draq5 (blue). (A, B) As compared with GFP-CDC control, *N1-ICD*-CDC yielded more cells that stained positively for smooth muscle actin; (C) the double-positive cells (yellow) identified *N1-ICD* modified CDC that had differentiated into cardiomyocytes; (D) cTnI staining for the non-infarct zone as a staining control; (E) the double-positive cells identified *N1-ICD* modified CDC that had differentiated into endothelial cells. MI, myocardial infarction. Color images available online at www.liebertonline.com/scd

Discussion

The results presented here demonstrate that Notch1 activation promotes differentiation of CDC into SMC in vitro in an *RBPJ*-dependent fashion. An injection of *N1-ICD*-expressing CDC into the ischemic myocardium yields enhanced numbers of implanted cells differentiating into SMC in vivo. To the best of our knowledge, this is the first study examining Notch-mediated CDC differentiation.

CDC constitute a heterogeneous population of progenitors with potential for heart regeneration. To identify the profile of CDC differentiation induced by constitutive *Notch-1* activation, we performed reverse transcription (RT)-PCR array analysis of terminal differentiation markers spanning 13 organs and tissues. We observed significant increases in SMC specific mRNA and protein expression in *N1-ICD*-CDC. Recently, *Notch1* has been reported to modulate cell differentiation in the proepicardium and adjacent pericardial mesoderm, and to be required for coronary vessel formation and myocardial growth during embryonic heart development [27]. Since recent reports indicate that epicardial tissue is a major source of CPC, in this regard, our data support a role for *Notch1* in endogenous cardiac progenitor commitment to SMC differentiation in coronary vessel formation. Pathophysiologically, a similar mechanism could be operative during vessel regeneration in ischemic heart disease. Recent studies have shown that activated CPC can regenerate conductive, small, and intermediate-sized arteries and capillary structures

and improve cardiac function in chronically injured rat hearts long after infarction and scar formation [6,7].

Data are conflicting regarding SMC differentiation of *Notch1* activated cells. Several in vitro studies indicated that *Notch1* signaling inhibits SMC differentiation of C3H10T1/2 fibroblasts [28] and human aortic SMC [29]. On the other hand, recent reports show that *Notch1* activation promotes SMC differentiation of human aortic endothelial cells and primary human foreskin fibroblasts via *RBPJ* directly binding to the alpha SMA promoter [17,30]. Likewise, we observed that SMC differentiation is *RBPJ* dependent. In *RBPJ* knockout CDC, the basal expression of SMC markers was significantly lower compared with the *RBPJ* floxed control CDC. Therefore, the role of *Notch1* in SMC differentiation may be cell specific. Our study specifically addresses the role of *Notch1* in differentiation of CDC, a promising cardiac progenitor source. We uncovered *Myh11* and *Tagln* as targets of Notch1 signaling via forced expression of the constitutively active *N1-ICD* in CDC.

Our in vivo experiments suggest that *N1-ICD* modified CDC yield considerably more implanted cells that express SMA, a marker of mature, differentiated SMC, suggesting a strong correlation between Notch activation and differentiation of implanted CDC into SMC. On the other hand, it is evident that not all implanted *N1-ICD*-CDC express SMA, suggesting that *N1-ICD* could regulate differentiation of CDC into other lineages in vivo and/or modulate cell survival, implantation, proliferation, or interactions with the micro-

environment. In addition, our experiments utilized adenoviral-mediated gene transfer, which produces transient expression of the transgene for a period of about 2 weeks. Sustained, as compared with transient, Notch activation could have profoundly different effects on the fate of implanted CDC. Further studies will be required to resolve these issues.

For the long-term cell culture over 78 days, we did not check the karyotype of the CDC, but we checked the possibility of cancer and/or cancer stem cells using CDC within 10 passages, and we did not observe obvious tumor growth at 4 months after an intramuscular injection in NOD/SCID mice, while we observed obvious tumor formation in mice injected with the same amount of mouse-induced pluripotent stem cells at 3 weeks after cell injection (Supplementary Fig. S2); thus, it seems that the possibility of cancer stem cells or cell line in CDC within 10 passage is low.

From our understanding of stem cell biology, *Notch-1* regulation of SMC differentiation of CDC has potentially far-reaching implications. After myocardial infarction, the vasculature in the infarct zone is impaired [31–33], which is directly related to survival [31]. Therefore, reestablishing blood flow to the damaged area is a critical step in restoring oxygen and nutrient supply to the cardiac matrix. Our data suggest that *Notch1* activation promotes the SMC commitment of CDC in vitro. Further studies examining the long-term effect of *Notch1/RBPJ* activation in CDC differentiation and postinfarction angiogenesis are warranted.

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Author Disclosure Statement

There is no conflict of interest in connection with this article.

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